0026-895X/03/6301-2–8$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:2–8, 2003

Vol. 63, No. 1
2075/1033631
Printed in U.S.A.

ACCELERATED COMMUNICATION

Tonically Activated $GABA_A$ Receptors in Hippocampal Neurons Are High-Affinity, Low-Conductance Sensors for Extracellular GABA

JACKY Y. T. YEUNG, KEVIN J. CANNING, GUOYUN ZHU, PETER PENNEFATHER, JOHN F. MACDONALD, and BEVERLEY A. ORSER

Institute of Medical Science (J.Y.T.Y., B.A.O.) and Departments of Anesthesia (K.J.C., B.A.O.), Physiology (G.Z., J.F.M, B.A.O.), and Pharmaceutical Sciences (P.P, J.F.M.), University of Toronto, Toronto, Ontario, Canada; and Department of Anesthesia, Sunnybrook and Women's College Health Science Center, Toronto, Ontario, Canada (B.A.O.)

Received August 12, 2002; accepted October 9, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

In the hippocampus, two distinct forms of GABAergic inhibition have been identified, phasic inhibitory postsynaptic currents that are the consequence of the vesicular release of GABA and a tonic conductance that is activated by low ambient concentrations of extracellular GABA. It is not known what accounts for the distinct properties of receptors that mediate the phasic and tonic inhibitory conductances. Moreover, the physiological role of the tonic inhibitory conductance remains uncertain because pharmacological tools that clearly distinguish tonic and phasic receptors are lacking. Here, we demonstrate that $GABA_A$ receptors that generate a tonic conductance in cultured hippocampal neurons from embryonic mice have different pharmacological properties than those in cerebellar granule neurons or pyramidal neurons in the dentate gyrus. The tonic conductance in cultured hippocampal neurons is enhanced by the benzodiazepine, midazolam, and is insensitive to the inhibitory effects of the competitive antagonist, gabazine ($\leq$10 μM). We also identify penicillin as an uncompetitive antagonist that selectively inhibits the synaptic but not tonic conductance. GABA was applied to hippocampal neurons to investigate the properties of synaptic and extrasynaptic receptors. GABA-evoked current was composed of two components: a rapidly desensitizing current that was blocked by penicillin and a nondesensitizing current that was insensitive to penicillin blockade. The potency of GABA was greater for the penicillin-insensitive nondesensitizing current. Single-channel studies show that the gabazine-insensitive $GABA_A$ receptors have a lower unitary conductance (12 pS) than that estimated for synaptic receptors. Thus, specialized $GABA_A$ receptors with an apparent higher affinity for GABA that do not readily desensitize mediate the persistent tonic conductance in hippocampal neurons. The receptors underlying tonic and phasic inhibitory conductances in hippocampal neurons are pharmacologically and biophysically distinct, suggesting that they serve different physiological roles.

The inhibitory neurotransmitter GABA is thought to regulate point-to-point communication between neurons by activating $GABA_A$ receptors clustered in postsynaptic densities. However, GABA also serves a "paracrine" function by diffusing away from the synaptic cleft and activating extrasynaptic $GABA_A$ and $GABA_B$ receptors that reside beyond subsynaptic domains (for review, see Isaacson, 2000; Mody, 2001). Ambient GABA can also arise by the reverse operation of GABA cotransporters in neurons and astrocytes (Liu et al., 2000; Wu et al., 2001). Thus, $GABA_A$ receptors need not be restricted to synapses to serve physiological functions. Moreover, $GABA_A$ receptors responsible for the tonic inhibitory conductance in some brain regions may be of clinical importance as targets for anesthetics and sedative drugs (Bai et al., 2001). Abnormal regulation of the tonic conductance may also play a role in hyperexcitatory disorders such as epilepsy. Anticonvulsants that increase the extracellular concentration of GABA may primarily increase the tonic inhibitory conductance (Overstreet and Westbrook, 2001). Despite their

This work was supported by the Ontario Ministry of Health, Epilepsy Canada, Canadian Institutes of Health Research, and Natural Sciences and Engineering Research Council of Canada.

ABBREVIATIONS: mIPSC, miniature inhibitory postsynaptic current; GBZ, gabazine; BIC, bicuculline; PEN, penicillin-G; VGB, vigabatrin; TBPS, *tert*-butyl-bicyclo[2.2.2]phosphorothionate; MIDZ; midazolam.

probable importance, the physiological role of tonic $GABA_A$ receptors in specific brain regions, including the hippocampus, remains to be elucidated.

At least 19 different $GABA_A$ subunits that confer distinct pharmacological and biophysical properties have been identified (Rudolph et al., 2001). Subunit composition influences agonist affinity, receptor kinetics, and segregation of receptors to subcellular regions of the neuron (Nusser et al., 1998). For example, extrasynaptic $GABA_A$ receptors in cerebellar granule neurons contain α_6 and δ subunits. The properties of native and recombinant $GABA_A$ receptors suggest that these subunits confer a high affinity for GABA, low single-channel conductance, and slow kinetics of desensitization (Saxena and Macdonald, 1994; Brickley et al., 1999; Haas and Macdonald, 1999; Mellor et al., 2000). Receptors containing δ subunits may also underlie a benzodiazepine-insensitive tonic conductance in dentate granule neurons (Nusser and Mody, 2002). Tonic inhibitory conductances have also been identified in the thalamus, the CA1 hippocampal region, and the cortex (Valeyev et al., 1998; Liu et al., 2000; Bai et al., 2001). However, the subunit composition and the pharmacological characteristics of $GABA_A$ receptors responsible for the tonic GABAergic conductance (referred to here as tonic receptors) remain to be elucidated in these brain regions.

We first showed that the tonic and synaptic conductances in embryonic hippocampal neurons displayed different sensitivities to the high-affinity competitive antagonist gabazine (SR-95531) (Bai et al., 2001). Also, noise analysis indicated the tonic receptors have a lower unitary channel conductance (7 pS) than that reported for synaptic receptors (25 pS). Here, we further characterized the properties of the tonic and synaptic (phasic) receptors in hippocampal neurons and identified penicillin-G as a selective antagonist of phasic receptors. Single-channel studies provided evidence that low-conductance, high-affinity channels mediated the tonic conductance.

Materials and Methods

Cell Culture and Electrophysiological Techniques. Cultures of hippocampal neurons were prepared from embryonic Swiss White mice as described previously (MacDonald et al., 1989). Conventional whole-cell currents were recorded under voltage-clamp (−60 mV) using an Axopatch 200 amplifier (Axon Instruments Inc., Union City, CA) that was interfaced to a Digidata 1200 (Instrutech Corp., Elmont, NY). Records were filtered (2 kHz) and digitized at 10 kHz using pClamp6 software (Axon Instruments Inc.) for off-line analyses.

Extracellular solutions contained 140 mM NaCl, 1.3 mM $CaCl_2$, 5.4 mM KCl, 25 mM HEPES, and 28 mM glucose, with pH adjusted to 7.4 using 1 M NaOH. Tetrodotoxin (300 nM) was added to the extracellular solution to block voltage-sensitive Na^+ channels, whereas 6-cyano-2,3-dihydroxy-7-nitroquinoxaline (10 μM) and 2-amino-5-phosphonovalerate (40 μM) were added to inhibit ionotropic glutamate receptors. Recording electrodes were filled with a solution containing 140 mM CsCl, 10 mM HEPES, 11 mM EGTA, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 4 mM MgATP, and 2 mM TEA, with the pH adjusted to 7.3 using CsOH. Control and drug-containing solutions were delivered to the cultured neurons through glass barrels that were positioned close to the somata.

Data Analysis. Spontaneous mIPSCs were analyzed using Mini-Analysis Software (Synaptosoft, Leonia, NJ) with the detection threshold set three times higher than the level of baseline noise. The peak amplitude, charge transfer (Q, determined by integrating the area under the mIPSCs), and time constant of current decay (τ_{decay}) were analyzed. The τ_{decay} was determined using the biexponential equation, $I(t) = A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, where I is the current amplitude at any given time (t), A_1 and A_2 are the amplitudes of the fast and slow decay components, and τ_1 and τ_2 are their respective decay time constants. The weighted time constant of current decay was determined by the equation $\tau_{decay} = \Sigma\ A_i\tau_i/\Sigma_{Ai}$.

Tonic current amplitude was calculated as the difference in the holding current measured before and after bicuculline methiodide (bicuculline, 10 μM) (Brickley et al., 1996; Wall and Usowicz, 1997). The holding current was analyzed using 15 100-ms segments that lacked mIPSCs. The segments were acquired before and after drug treatment. All-point histograms were constructed from these segments and mean values were determined from Gaussian fits (pStat; Axon Instruments Inc.). The root-mean-square noise (σ) was calculated from segments that lacked mIPSCs using Mini Analysis software (Synaptosoft) according to the equation $\sigma = \sqrt{\Sigma(x_i - \text{mean } x)^2/n}$, where x_i represents each individual point, mean x represents the average of all points, and n represents the total number of points (5120 points).

Single Channel Analysis. Outside-out patches were excised from the somata and voltage clamped at −70 mV. Recordings were low-pass–filtered at 1 kHz (−3 dB, 8-pole Bessle), digitized at 20 kHz, and recorded on videotape for off-line analysis. To estimate the amplitude of the various conductance levels, we first constructed all-point histograms. The current-voltage relationships of the various conductance levels were also analyzed using the all-point histograms. Amplitude and all-point histograms were fitted with the sum of several Gaussian components using a Levenberg-Marguardt least-squares minimization (pStat; Axon Instruments Inc.). The frequency of channel opening was determined using the threshold crossing method. High-, mid-, and low-conductance openings that crossed the 50% threshold level were selected and the minimal open duration was set at 200 μs.

Results

Tonic and Synaptic $GABA_A$ Receptors Are Pharmacologically Distinct. Two distinct types of current were evident in whole-cell recordings, including phasic mIPSCs and a persistent tonic current that was revealed by the application of the $GABA_A$ receptor antagonist, bicuculline (Fig. 1A). The mIPSCs had a frequency of 2.1 ± 0.7 Hz, a rise time of 2.8 ± 0.3 ms, an amplitude of 27.2 ± 2.6 pA (n = 6), and a decay that was best described by a biexponential equation with a weighted time constant (t_{decay}) of 19.3 ± 2.9 ms. Bicuculline (10 μM) abolished the mIPSCs and produced an outward shift in the baseline by 18.4 ± 1.3 pA (n = 39). Higher concentrations of bicuculline (100 μM) caused no further shift in the holding current. The tonic conductance was mediated by $GABA_A$ receptors as the current reversed polarity at the chloride equilibrium potential and was blocked by other $GABA_A$ receptor antagonists (Bai et al., 2001). Bicuculline (10 μM) also reduced the baseline noise as indicated by a reduction in the root-mean-square value (σ) from 4.2 ± 0.2 to 2.9 ± 0.1 pA, (n = 39, $p < 0.05$). The ratio of the variance/mean tonic current ($[\sigma^2_{+tonic} - \sigma^2_{-tonic}]/I_{tonic}$) predicted the unitary conductance of the underlying tonic receptors to be 8 pS. This value is smaller than the full conductance state estimated for synaptic receptors (25 pS; De Koninck and Mody, 1994) but similar to the low-conductance state observed in the single-channel studies described below.

The high-affinity antagonist gabazine (≤10 μM) abolished the mIPSCs but failed to reduce the amplitude of the tonic current (1.1 ± 2.9 pA, n = 16), as we reported previously (Bai

et al., 2001). This apparent insensitivity to gabazine could result from a higher affinity of the tonic receptors for GABA (Stell and Mody, 2002) or from the greater potency of gabazine for synaptic receptors. Indeed, higher concentrations of gabazine (≥100 μM) reduced the holding current (6.3 ± 10.0 pA, $n = 6$) and the σ (3.9 ± 0.5 to 2.9 ± 0.2 pA, $n = 6$) indicating that gabazine modulates tonic receptor function. Furthermore, although gabazine is considered to be a competitive antagonist, it also allosterically regulates $GABA_A$ receptor activity (Ueno et al., 1997). We previously proposed that gabazine may act as a partial agonist at the $GABA_A$ receptors in embryonic hippocampal neurons to activate a small residual conductance (Bai et al., 2001). Consistent with this prediction, in three recordings, gabazine (100 μM) caused a small inward current (13.5 ± 5.4 pA) that was blocked by bicuculline (10 μM). Thus, gabazine blocked synaptic receptors but also enhanced the tonic current, making it a less than an ideal tool for probing the physiological roles of the tonic conductance.

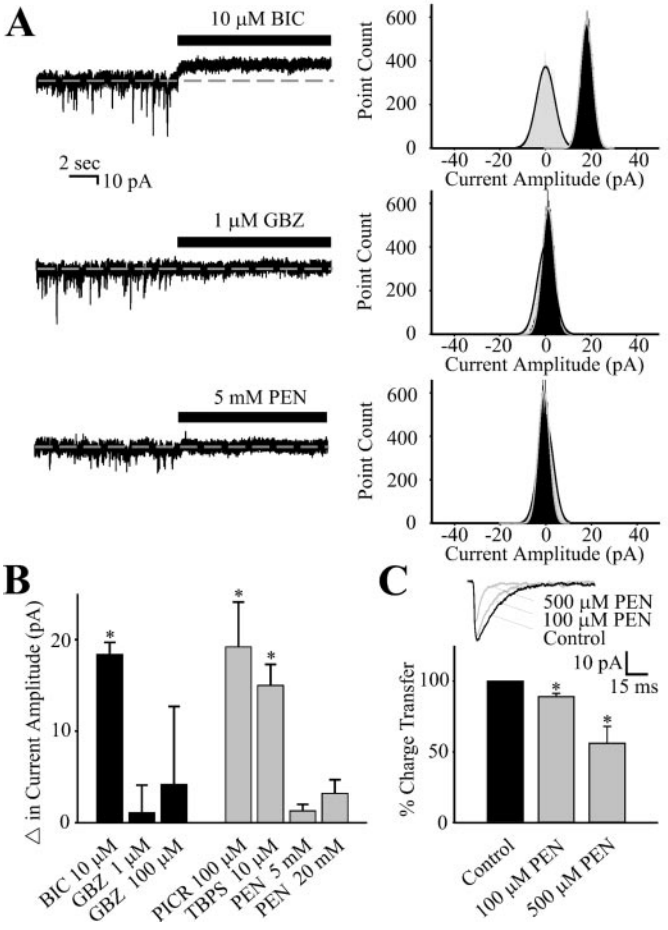

Fig. 1. Antagonist sensitivity of tonic and phasic conductances. A, currents were recorded before and after bicuculline (BIC), gabazine (GBZ), or penicillin (PEN). The dashed gray line indicates the amplitude of the baseline current before drug application. Corresponding all-point histograms are also shown to the right. The gray and black histograms illustrate the amplitude of the holding current in the absence and presence of the antagonist, respectively. Note that BIC caused a rightward shift of the histogram, consistent with a reduction in current amplitude, whereas the histograms for GBZ and PEN overlap control because there was no change in the holding current. B, the histograms illustrate that BIC ($n = 39$, $p < 0.05$), picrotoxin (PICRO, $n = 4$, $p < 0.05$), and TBPS ($n = 4$, $p < 0.05$) but not PEN (5 mM, $n = 25$; 20 mM, $n = 6$) or GBZ (1 μM, $n = 16$; 100 μM, $n = 6$) reduce the amplitude of the tonic current. C, the effect of PEN 100 and 500 μM on mIPSCs are illustrated. Charge transfer (Q) was reduced by 100 μM and 500 mM PEN to 89.0 ± 2.3 and 56.1 ± 11.9% of control values ($n = 4$, $p < 0.05$), respectively.

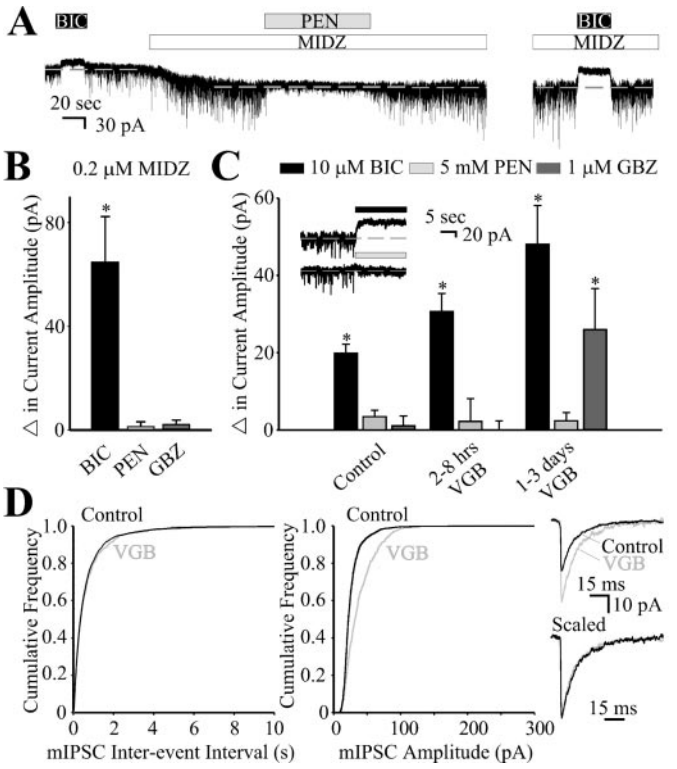

Fig. 2. Midazolam (MIDZ) and vigabatrin (VGB) enhanced the tonic current. A and B, MIDZ (0.2 μM) increased the tonic current ~3.5 fold. BIC (10 μM, $n = 3$) but not PEN (5 mM, $n = 4$) or GBZ (1 μM, $n = 3$) reduced the holding current. Note that BIC (10 μM) failed to reduce the MIDZ-enhanced current to the baseline level. In one of three recordings, a higher concentration of bicuculline (100 μM) was required to reduce the holding current to that observed in the absence of midazolam. C, the tonic current was enhanced after treatment with VGB for 2 to 8 h and 1 to 3 days by 54 and 142%, respectively ($p < 0.05$). The VGB-enhanced current was insensitive to PEN (5 mM) but not GBZ (1 μM). The inset illustrates BIC and PEN effects on tonic current. D, the amplitude of the mIPSCs was increased by VGB after 1 to 3 days but not 2 to 8 h. Cumulative histograms show the frequency and amplitude of mIPSCs before ($n = 10$) and after VGB treatment ($n = 6$, $p < 0.05$). Right, mIPSC from control and treated neurons (2 days). The lower traces show control normalized to peak VGB-enhanced mIPSC).

Selective Blockade of Synaptic Receptors by Penicillin. In a continued search for compounds that distinguish between tonic and phasic conductances, we examined the effects of the noncompetitive antagonists picrotoxin, *tert*-butyl-bicyclo[2.2.2]phosphorothionate (TBPS), and penicillin-G (penicillin). Rather than compete at the GABA recognition site, these antagonists block the receptor by binding to sites within or near the open channel pore. TBPS (10 μM) and picrotoxin (100 μM) abolished the mIPSCs and blocked the tonic current as evidenced by the outward shift of the baseline (15.0 ± 2.3 pA, $n = 4$, and 19.2 ± 4.9 pA, $n = 4$, respectively; Fig. 1B). In contrast, penicillin (0.1 to 20 mM) caused a concentration-dependent inhibition of the synaptic currents but failed to block the tonic current (Fig. 1C). Although the amplitude of the tonic current was unchanged by penicillin (5 mM; 1.3 ± 0.7 pA, $n = 25$), σ was slightly reduced (3.9 ± 0.3 to 3.1 ± 0.2 pA; $p < 0.05$). Higher concentrations of penicillin (20 mM) had no additional action on the amplitude of the tonic current (3.2 ± 1.5 pA, $n = 6$, $p > 0.05$);

however, it further reduced σ (4.4 ± 0.8 to 3.2 ± 0.4 pA, $p < 0.05$). These results suggest that although penicillin did not block the tonic current, it nevertheless influences the tonic receptors. This action is expected for an uncompetitive blocker that fails to block responses generated under conditions of low receptor occupancy (Pennefather and Quastel, 1982).

Enhanced Tonic Receptor Function and Penicillin Blockade. Activation of the $GABA_A$ receptor facilitates inhibition by use-dependent antagonists that require channel opening to reach their site of blockade. Thus, the relative insensitivity of tonic receptors to penicillin could result from a low probability of channel opening. Two strategies were used to determine whether an increase in channel opening influenced the penicillin sensitivity of the tonic receptors. First, the benzodiazepine midazolam was applied to allosterically enhance $GABA_A$ receptor activity. Midazolam (0.2 μM) increased the amplitude of the tonic current by 3.5-fold (64.2 ± 18 pA, $n = 3$, $p < 0.05$; Fig. 2B). Despite this enhancement, penicillin (5 mM, 1.4 ± 1.8 pA, $n = 4$; Fig. 2B) and gabazine (1 μM, 2.2 ± 1.6 pA, $n = 3$; Fig. 2B) failed to reduce the tonic current. Next, to increase the ambient concentration of GABA, the degradation of GABA by GABA-transaminase was inhibited by the GABA-transaminase antagonist vigabatrin (Engel et al., 2001). Cultures were treated with vigabatrin (100 μM) for various time intervals (2 h to 3 days) then washed before the recordings.

Vigabatrin (2–8 h) increased the tonic current by 54% (30.7 ± 4.6 pA, $n = 9$, $p < 0.05$; Fig. 2C), whereas the σ was unchanged (4.5 ± 1.0 pA, $n = 9$, $p > 0.05$). After 1 to 3 days, the tonic current was increased by 142% (48.1 ± 10.0 pA, $n = 11$ versus 19.9 ± 2.3 pA in control cultures, $n = 10$, $p < 0.05$) and the noise increased proportionally (6.1 ± 1.9 pA, $p < 0.05$, $n = 11$; control 4.6 ± 0.9 pA, $n = 10$, $p < 0.05$). The variance/mean tonic current ratio predicted a channel conductance of 9.4 pS, supporting the hypothesis that low-conductance channels generate the tonic current.

Despite an increase in the tonic current after vigabatrin (2–8 h), the amplitude, time course, and frequency of mIPSCs were unchanged: 30.9 ± 1.5 pA, $n = 4$, control, 27.2 ± 2.6 pA; $n = 6$; 10-to-90% rise time, 2.5 ± 0.1 ms; control, 2.8 ± 0.3 ms; and τ_{decay}, 18.8 ± 1.9 versus 19.3 ± 2.9 ms, respectively. Thus, factors that regulate GABA metabolism influenced the tonic and synaptic currents differently. After the prolonged treatment (1–3 days), the amplitude of mIPSCs was increased 49% ($n = 6$, $p < 0.05$), whereas the frequency, rise time, and decay were unchanged: 1.5 ± 0.5 Hz; 10-to-90% rise time, 2.4 ± 0.3 ms; τ_{decay}, 19.1 ± 1.0 ms, $n = 6$ (Fig. 2D). These results are consistent with a report indicating a long-term application (4 days) of vigabatrin increased the amplitude of mIPSCs in rat hippocampal slices (Engel et al., 2001). They also suggest that the content of GABAergic vesicles is increased after a reduction in GABA breakdown (Loscher et al., 1986). The rise in cytosolic concentration of GABA may also enhance the reverse operation of GABA transporters, leading to an increase in ambient GABA.

Despite the enhancement by vigabatrin, the tonic conductance remained insensitive to penicillin blockade (Fig. 2C). Penicillin (5 mM) abolished the mIPSCs but failed to reduce the tonic current (2–8 h, 2.2 ± 5.9 pA, $n = 5$; 1–3 days, 2.3 ± 2.1 pA, $n = 5$ versus control, 3.4 ± 1.7 pA, $n = 6$). Similarly, gabazine (1 μM) failed to block the tonic current after the 2- to 8-h treatment (1.1 ± 2.5 pA, $n = 8$; 2 to 8 h, 0.0 ± 2.3 pA, $n = 5$). In cells treated for 1 to 3 days, gabazine reduced the tonic current to control levels (26.0 ± 10.6 pA, $p < 0.05$, $n = 7$). This effect is expected if gabazine displaces GABA and acts as a partial agonist. Together, the above findings show that the tonic receptor is relatively insensitive to penicillin blockade despite an increase in channel opening.

GABA-Evoked Current Reveals Two Populations. If cultured hippocampal neurons contain two distinct populations of $GABA_A$ receptors (tonic penicillin-*insensitive* and phasic penicillin-*sensitive* receptors), then it is predicted that exogenous GABA would generate currents composed of two components. Also, at least two previous reports suggest that synaptic receptors mediate the fast component of GABA-evoked current, whereas extrasynaptic receptors mediate the slow deactivating and desensitizing component (Bai et al., 1999; Banks and Pearce, 2000).

Consistent with this prediction, the peak current, activated by GABA, was inhibited by penicillin (1 mM by 82.4 ± 2.8%, $n = 9$, $p < 0.05$) whereas the steady-state current was relatively insensitive to block (−9.5 ± 2.6%, $n = 9$; Fig. 3, A and B). In some recordings in which mIPSCs were seen superimposed on the GABA-evoked current, penicillin abolished the mIPSCs but failed to reduce the steady-state current (Fig. 3A, inset). Penicillin also failed to inhibit the non-desensitizing current evoked by a low concentration of GABA (1 μM).

Next, we predicted that if high- and low-affinity receptors

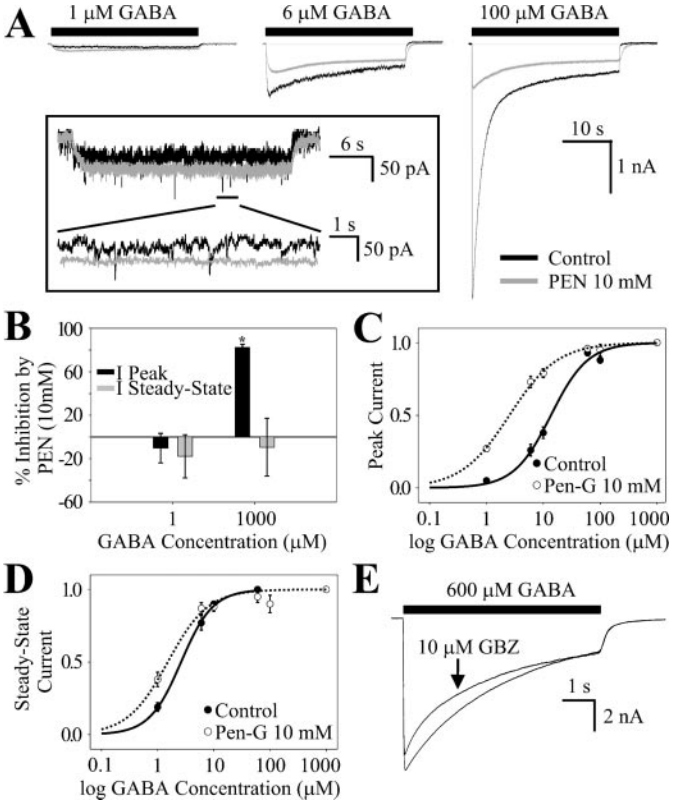

Fig. 3. Currents are composed of PEN-sensitive and -insensitive components. A, PEN (gray) inhibited the peak but not steady-state current (inset). mIPSCs superimposed on the GABA-evoked current were blocked by PEN. B, PEN blocked the peak but not steady-state current. C and D, concentration-response plots in the absence (solid line) and presence (broken line) of PEN show PEN increased the potency of GABA. E, GBZ preferentially blocked the peak current.

underlay the tonic and synaptic conductances, respectively, then the potency of GABA would be greater for the penicillin-insensitive current compared with the penicillin-sensitive current. To determine the potency of GABA, concentration-response plots were constructed for currents recorded in the absence and presence of penicillin (10 mM; Fig. 3, C and D). The concentration that activated 50% of the maximal current (EC_{50}) was reduced 5-fold by penicillin from 13.6 to 2.7 μM ($n = 9$, $p < 0.05$) whereas the EC_{50} value of the steady-state current was reduced 1.8-fold from 2.8 to 1.6 μM ($n = 9$, $p < 0.05$). Similar to penicillin, gabazine preferentially inhibited the peak compared with the steady-state current (Fig. 3E). The concentrations of gabazine that inhibited 50% (IC_{50}) of the peak and steady-state current evoked by GABA (600 μM) were 20.7 ± 1.57 μM (Hill coefficient, 1.41 ± 0.14; $n = 5$) and 34.9 ± 4.9 μM (Hill coefficient, 1.71 ± 0.37, $n = 5$), respectively. Thus, the potency of GABA was increased in the presence of penicillin for both the rapidly desensitizing peak current (2.7 versus 13.6 μM) and the steady-state current (1.6 and 2.8 μM).

Low Conductance Tonic Receptors. Information about the conductance of tonic receptors has been limited to estimates obtained from noise analysis. In a previous study, we showed the tonic receptors in hippocampal neurons had a lower conductance than that estimated from nonstationary fluctuation analysis of synaptic receptors (Bai et al., 2001). To investigate the unitary conductance of tonic receptors, we recorded single-channel activity from outside-out patches excised from the somata. GABA (0.5–2 μM) activated bicuculline-sensitive (2 μM) channel openings that displayed at least three discrete conductance levels. The chord conductance values of the high-, mid-, and low-conductance levels were 28.7 ± 0.5 pS, 20.7 ± 0.5 pS, and 12.3 ± 0.4 pS, respectively ($n = 14$; Fig. 4A). The three open levels did not require low concentrations of GABA because they were also evident in patches exposed to 50 μM GABA (29.4 ± 0.4, 21.7 ± 0.3, and 13.9 ± 0.4 pS, $n = 7$).

To determine the relative open probabilities (P_{open}) of the three conductance levels, all-point histograms were generated and fit with multiple Gaussian components. The area under each Gaussian peak was used to estimate the relative occurrence of events at the corresponding amplitude level. The relative P_{open} values of the high-, mid-, and low-conductance levels were 0.12 ± 0.026, 0.07 ± 0.009, and 0.087 ± 0.012, respectively ($n = 6$). To ensure that the openings were mediated by $GABA_A$ receptors, midazolam (0.1 μM) was applied and the relative P_{open} was again determined. Midazolam increased P_{open} of the high-, mid-, and low-conductance levels to 353 ± 73, 266 ± 60, and 238 ± 45% ($n = 3$, $p < 0.05$) of control, respectively. This effect of midazolam was reduced by flumazenil (1 μM, P_{open} = 167 ± 50, 155 ± 8, and 139 ± 1% of control, $n = 3$).

To identify the single-channel conductance of the tonic receptors, we applied gabazine (2 μM) at a concentration that failed to block the tonic conductance in whole-cell recordings (Fig. $4B_1$). The effect of penicillin on the various conductance levels was not examined as penicillin causes a rapid blockade of $GABA_A$ receptors that reduces the apparent amplitude of single channel openings (Chow and Mathers, 1986). Two approaches were used to analyze channel openings before and after gabazine (2 μM). First, P_{open} was determined by analyzing the relative areas of the all-point histograms. Next, the relative frequency of channel opening was measured by counting the number of events at each open level (Fig. $4B_2$). Both methods yielded qualitatively similar results. Gabazine (2 μM) changed the open probability of the 29.3-, 20.4-, and 12.3-pS channels by 26 ± 6.4, 40 ± 7.9, and 125 ± 14% ($n = 6$, $p < 0.05$) of control values, respectively. It was not possible to determine whether the increase in P_{open} of the low conductance level by gabazine resulted from the "unmasking" of previously obscured low-conductance openings or from a shift in opening to a low subconductance state. An analysis of the frequency of channel levels (Fig. $4B_2$) confirmed the relative insensitivity of the low-conductance openings to blockade by gabazine.

Discussion

Selective Inhibition by Penicillin and Gabazine. Herein, we confirm that the tonic and synaptic conductances in embryonic hippocampal neurons have different sensitivi-

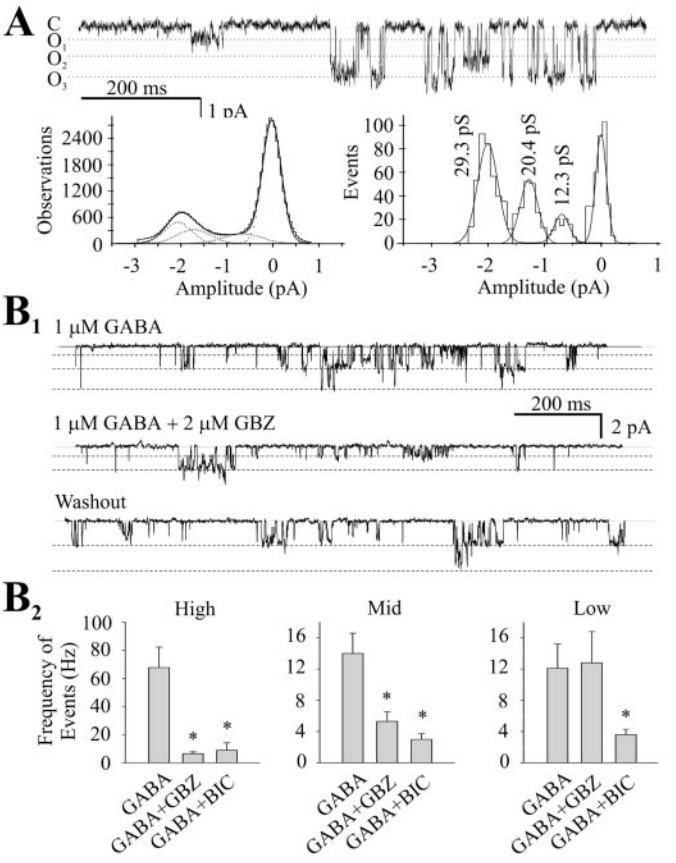

Fig. 4. GABA-gated single channel openings in outside-out patches from cultured hippocampal neurons. A, at least three discrete conductance levels are evident for currents activated by GABA (1 μM). The dashed lines indicate the amplitude of the low-, mid-, and high-conductance levels (O_1, O_2, O_3, respectively). All-point (left) and amplitude (right) histograms are shown where the all-point histograms were fit with the sum of multiple Gaussian components as indicated by the dotted lines. The solid line indicated the sum of the individual components. The amplitude histogram indicates the mean conductance values were 29.3, 20.4, and 12.3 pS. B_1, GBZ (2 μM) blocked the high- and mid-conductance openings but failed to inhibit the low-conductance events. B_2, the frequency histograms indicate the number of opening per second (Hz) of the various conductance levels in the presence of GBZ (2 μM) or BIC (10 μM). BIC reduced the frequency of all levels to a similar extent (n = 5–6 patches), whereas GBZ preferentially inhibited the high- and mid-conductance openings.

ties to gabazine (10 μM). This finding contrasts the nonselective inhibition by gabazine (10 μM) of tonic and synaptic conductances in cerebellar granule neurons and dentate gyrus (Brickley et al., 2001; Hamann et al., 2002; Stell and Mody, 2002). The tonic conductance in cultured hippocampal neurons also differs from that in the dentate gyrus and the cerebellum in that it is sensitive to benzodiazepines, which implies that the underlying receptors in cultured hippocampal neurons contain γ subunits but lack δ subunits.

The agonist affinity is critically dependent on the subunit composition of $GABA_A$ receptors. Different subunit compositions and hence GABA affinity may account for differences in the sensitivities of the tonic conductance to competitive antagonists in the various brain regions. Also, the concentration and time course of GABA that activates tonic and synaptic conductances in the various experimental preparations probably differ. Thus, in addition to the intrinsic properties of the receptors, pharmacokinetic factors could also influence the sensitivity to competitive antagonists. Consequently, gabazine is not an ideal drug for discriminating between the tonic and synaptic currents. We next show that penicillin, a noncompetitive antagonist, selectively blocks synaptic but not tonic conductance in hippocampal neurons. We also characterize the effects of penicillin on synaptic and extrasynaptic $GABA_A$ receptors activated by exogenous GABA. In a previous study, GABA-evoked current in CA1 pyramidal neurons revealed an initial rapidly deactivating component that was attributed to synaptic receptors and a slowly deactivating component that was attributed to extrasynaptic receptors (Banks and Pearce, 2000). Another study also showed that GABA-evoked current in cultured hippocampal neurons was composed of rapidly and slowly desensitizing components (Bai et al., 1999). The pharmacological properties of the slowly desensitizing current differ from those of synaptic currents, suggesting that they are mediated by extrasynaptic receptors. Based on these studies, we predicted and observed that exogenous GABA would activate a low-affinity peak current that rapidly desensitized (putatively mediated by synaptic receptors) and a more slowly decaying, high-affinity component that did not desensitize (putatively mediated by extrasynaptic receptors). Furthermore, penicillin would preferentially block the peak but not steady-state current.

Distinct Subunit Composition or State-Dependent Differences in a Single Receptor Population. The critical question remains: do the different pharmacological properties of tonic and synaptic $GABA_A$ conductances in hippocampal neurons represent two receptor populations with unique subunit structures, are they merely state-dependent changes in a single population, or some combination of these two possibilities? Several lines of evidence suggest that the tonic and synaptic receptors in cultured neurons have different molecular structures. Our results indicate that penicillin caused a leftward shift in the GABA concentration-response plot. This finding contrasts reports that showed penicillin failed to increase the potency of GABA for recombinant $GABA_A$ receptors and native receptors in the rat frontal cortex (Sugimoto et al., 2002). It should also be noted, however, that an uncompetitive antagonist can cause a leftward shift in the concentration-response relationship, even for a single population of receptors (Pennefather and Quastel, 1982). However, an uncompetitive mechanism of blockade cannot fully account for our findings because the peak and steady-state currents were not influenced to the same extent. Furthermore, these results do not rule out the possibility that gabazine and penicillin preferentially blocked a high conductance state of the $GABA_A$ receptor.

The single-channel studies revealed multiple conductance levels that also represent either $GABA_A$ receptors with differing molecular structures or subconductance states of the same receptor population (Birnir et al., 2001). Consistent with a difference in the molecular structure, receptors with distinct molecular structures that mediate the tonic conductance in cerebellar granule neurons have a lower single-channel conductance compared with synaptic receptors (Brickley et al., 1999; Leao et al., 2000). Also, studies of recombinant subunits and native receptors indicate that γ_2-deficient receptors display a low channel conductance (Moss et al., 1990; Angelotti et al., 1992; Lorez et al., 2000). Alternatively, the multiple open levels of $GABA_A$ receptors in cultured hippocampal neurons may correspond to differentially liganded states associated with different conductance levels. Indeed, substrate-dependent gating was reported for $GABA_A$ receptors (Birnir et al., 2001), as was shown for glutamate-gate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid channels, in which single-channel conductance depends on the concentration of agonist (Smith and Howe, 2000). $GABA_A$ receptors have multiple binding sites for agonist, each with a different affinity for GABA. Channel conductance and sensitivity to antagonists may depend on the number of GABA binding sites occupied. For example, the low-conductance $GABA_A$ channel openings that were insensitive to gabazine blockade may represent a mono-liganded form of the channel.

The efficacy of GABA acting on receptors located at distances away from the presynaptic terminal would be enhanced if extrasynaptic receptors had a high affinity for GABA and desensitized slowly. This study demonstrates that specialized $GABA_A$ receptors with an apparent higher affinity for GABA that do not readily desensitize mediate the persistent tonic conductance in hippocampal neurons. Single-channel studies indicate that the concentration of ambient GABA (0.2–0.8 μM) in the brain is sufficient to activate low-conductance, gabazine-insensitive tonic receptors (Lerma et al., 1986). Penicillin failed to block both the tonic conductance and the nondesensitizing current activated by exogenous GABA, suggesting that the tonic receptors represent a subpopulation that fails to desensitize. It should be possible to exploit the differential effects of penicillin on tonic and phasic conductances to test the hypotheses that extrasynaptic and synaptic receptors in hippocampal neurons serve different physiological purposes and that they are structurally distinct.

Acknowledgments

We thank Drs. Michael F. Jackson and Yaël Perez for their review of this manuscript. We also thank Lidia Brandes and Ella Czerwinska for technical support.

References

Angelotti TP, Tan F, Chahine KG, and Macdonald RL (1992) Molecular and electrophysiological characterization of a allelic variant of the rat α6 GABAA receptor subunit. *Brain Res Mol Brain Res* **16:**173–178.

Bai D, Pennefather PS, MacDonald JF, and Orser BA (1999) The general anesthetic propofol slows deactivation and desensitization of $GABA_A$ receptors. *J Neurosci* **19:**10635–10646.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF, and Orser BA (2001)

Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric acid$_A$ receptors in hippocampal neurons. *Mol Pharmacol* **59:**814–824.
Banks MI and Pearce RA (2000) Kinetic differences between synaptic and extrasynaptic GABA$_A$ receptors in CA1 pyramidal cells. *J Neurosci* **20:**937–948.
Birnir B, Eghbali M, Cox GB, and Gage PW (2001) GABA concentration sets the conductance of delayed GABA$_A$ channels in outside-out patches from rat hippocampal neurons. *J Membr Biol* **181:**171–183.
Brickley SG, Cull-Candy SG, and Farrant M (1996) Development of a tonic form of synaptic inhibition in rat cerebellar granule cells resulting from persistent activation of GABA$_A$ receptors. *J Physiol (Lond)* **497:**753–759.
Brickley SG, Cull-Candy SG, and Farrant M (1999) Single-channel properties of synaptic and extrasynaptic GABA$_A$ receptors suggest differential targeting of receptor subtypes. *J Neurosci* **19:**2960–2973.
Brickley SG, Revilla V, Cull-Candy SG, Wisden W, and Farrant M (2001) Adaptive regulation of neuronal excitability by a voltage-independent potassium conductance. *Nature (Lond)* **409:**88–92.
Chow P and Mathers D (1986) Convulsant doses of penicillin shorten the lifetime of GABA-induced channels in cultured central neurones. *Br J Pharmacol* **88:**541–547.
De Koninck Y and Mody I (1994) Noise analysis of miniature IPSCs in adult rat brain slices: properties and modulation of synaptic GABA$_A$ receptor channels. *J Neurophysiol* **71:**1318–1335.
Engel D, Pahner I, Schulze K, Frahm C, Jarry H, Ahnert-Hilger G, and Draguhn A (2001) plasticity of rat central inhibitory synapses through GABA metabolism. *J Physiol* **535:**473–482.
Haas KF and Macdonald RL (1999) GABA$_A$ receptor subunit γ_2 and δ subtypes confer unique kinetic properties on recombinant GABA$_A$ receptor currents in mouse fibroblasts. *J Physiol (Lond)* **514:**27–45.
Hamann M, Rossi DJ, and Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* **33:**625–633.
Isaacson JS (2000) Spillover in the spotlight. *Curr Biol* **10:**R475–R477.
Leao RM, Mellor JR, and Randall AD (2000) Tonic benzodiazepine-sensitive GABAergic inhibition in cultured rodent cerebellar granule cells. *Neuropharmacology* **39:**990–1003.
Lerma J, Herranz AS, Herreras O, Abraira V, and Martin del Rio R (1986) In vivo determination of extracellular concentration of amino acids in the rat hippocampus. A method based on brain dialysis and computerized analysis. *Brain Res* **384:**145–155.
Liu QY, Schaffner AE, Chang YH, Maric D, and Barker JL (2000) Persistent activation of GABA$_A$ receptor/Cl$^-$ channels by astrocyte-derived GABA in cultured embryonic rat hippocampal neurons. *J Neurophysiol* **84:**1392–1403.
Lorez M, Benke D, Luscher B, Mohler H, and Benson JA (2000) Single-channel properties of neuronal GABA$_A$ receptors from mice lacking the γ_2 subunit. *J Physiol* **527 Pt 1:**11–31.
Loscher W, Jackel R, and Czuczwar SJ (1986) Is amygdala kindling in rats a model for drug-resistant partial epilepsy? *Exp Neurol* **93:**211–226.
MacDonald JF, Mody I, Salter MW, Pennefather P, and Schneiderman JH (1989) The regulation of NMDA receptors in the central nervous system. *Prog Neuropsychopharmacol Biol Psychiatry* **13:**481–488.
Mellor JR, Wisden W, and Randall AD (2000) Somato-synaptic variation of GABA$_A$ receptors in cultured murine cerebellar granule cells: investigation of the role of the α6 subunit. *Neuropharmacology* **39:**1495–1513.
Mody I (2001) Distinguishing between GABA$_A$ receptors responsible for tonic and phasic conductances. *Neurochem Res* **26:**907–913.
Moss SJ, Smart TG, Porter NM, Nayeem N, Devine J, Stephenson FA, Macdonald RL, and Barnard EA (1990) Cloned GABA receptors are maintained in a stable cell line: allosteric and channel properties. *Eur J Pharmacol* **189:**77–88.
Nusser Z and Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol* **87:**2624–2628.
Nusser Z, Sieghart W, and Somogyi P (1998) Segregation of different GABA$_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* **18:**1693–1703.
Overstreet LS and Westbrook GL (2001) Paradoxical reduction of synaptic inhibition by vigabatrin. *J Neurophysiol* **86:**596–603.
Pennefather P and Quastel DM (1982) Modification of dose-response curves by effector blockade and uncompetitive antagonism. *Mol Pharmacol* **22:**369–380.
Rudolph U, Crestani F, and Mohler H (2001) GABA$_A$ receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol Sci* **22:**188–194.
Saxena NC and Macdonald RL (1994) Assembly of GABA$_A$ receptor subunits: role of the δ subunit. *J Neurosci* **14:**7077–7086.
Smith TC and Howe JR (2000) Concentration-dependent substate behavior of native AMPA receptors. *Nat Neurosci* **3:**992–997.
Stell BM and Mody I (2002) Receptors with different affinities mediate phasic and tonic GABA$_A$ conductances in hippocampal neurons. *J Neurosci* **22:**RC223.
Sugimoto M, Fukami S, Kayakiri H, Yamazaki S, Matsuoka N, Uchida I, and Mashimo T (2002) The beta-lactam antibiotics, penicillin-G and cefoselis have different mechanisms and sites of action at GABA$_A$ receptors. *Br J Pharmacol* **135:**427–432.
Ueno S, Bracamontes J, Zorumski C, Weiss DS, and Steinbach JH (1997) Bicuculline and gabazine are allosteric inhibitors of channel opening of the GABA$_A$ receptor. *J Neurosci* **17:**625–634.
Valeyev AY, Schaffner AE, Skolnick P, Dunlap VS, Wong G, and Barker JL (1998) Embryonic rat hippocampal neurons and GABAA receptor subunit-transfected non-neuronal cells release GABA tonically. *J Membr Biol* **164:**239–251.
Wall MJ and Usowicz MM (1997) Development of action potential-dependent and independent spontaneous GABA$_A$ receptor-mediated currents in granule cells of postnatal rat cerebellum. *Eur J Neurosci* **9:**533–548.
Wu Y, Wang W, and Richerson GB (2001) GABA transaminase inhibition induces spontaneous and enhances depolarization-evoked GABA efflux via reversal of the GABA transporter. *J Neurosci* **21:**2630–2639.

Address correspondence to: Dr. B.A. Orser, Department of Physiology, Medical Science Building, Room 3318, University of Toronto, 1 King's College Circle, Toronto, Ontario M5S-1A8, Canada. E-mail: beverley.orser@utoronto.ca